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BODY CONSTRAINT AND DEVELOPMENTAL ARREST IN *GALLERIA MELLONELLA* L.: FURTHER STUDIES

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The sequence of events in insect development that follows activation of the prothoracic glands and culminates in molting or metamorphosis is now well established but the mechanism by which the sequence is initiated at the appropriate time and place remains little understood. It is reasonable to suppose that several or many requirements, such as nutritional state, location, and appropriate body form should be met before a larva molts, or enters the developmental crisis of pupation. If integrated information about these factors converges upon neurosecretory cells, either to activate them, or release them from inhibition, it should be possible to delay release of neurosecretory material, and thus suspend development. Until the neural pathways to the neurosecretory cells of the brain are worked out, evidence of the mechanism of release can only be circumstantial: it must be shown in each case that a manipulation which suspends development is not acting at a point in the sequence beyond prothoracotropic hormone release. The capacity of implanted endocrine organs, or injected hormone to override developmental arrest provides suggestive, but not conclusive information about mechanisms of arrest.

Treatments which may be expected to alter sensory input to the brain of *Galleria mellonella*, such as severing the ventral nerve cord or bodily constraint, prevent initiation of the larval-pupal molting cycle and it has been suggested that these operations may inhibit the release of prothoracotropic hormone as a result of modified proprioreceptive input (Edwards, 1967). Such treatments might in fact intervene in the molt controlling mechanisms at several levels: (a) by inhibiting the secretion of prothoracotropic hormone from the brain, (b) by blocking the response of the prothoracic glands to the hormone, or otherwise preventing the secretion of molting hormone, or (c) by directly affecting the capacity of epidermis to respond to the hormones.

The aim of this study was to limit these possibilities. It is shown that restraint neither blocks the capacity of the prothoracic glands to secrete ecdysone, nor the capacity of the epidermis to respond to ecdysone. The mechanism is thus limited to inhibition of brain activity. The precise means by which prothoracotropic hormone is withheld remains to be demonstrated, but the results are consistent with the hypothesis that modification of sensory input underlies the inhibition, and that least in the wax moth, body form is a significant factor.

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Material and Methods

Wax moth larvae of known age were used for all experiments. Larvae were grown on an artificial medium (Sehnal and Schneiderman, in preparation) at 30° C and 60–80% R.H. Larvae were restrained as previously described (Edwards, 1967) using Scotch brand "Magic Mending" tape No. 810. They were checked twice daily, and occasional escapees were again restrained. As in work reported earlier (Edwards, 1967) the adhesive tape was applied in such a way as to insure that heart beat and blood circulation persisted. The restraining tape did not act as a ligature. The larvae spun silk and excreted a dry paste of urate material. Up to three spiracles were occluded by adhesive tape, but oxygen deprivation does not appear to be significant in these experiments for local sealing of spiracles with wax does not suspend development. Longitudinal tracheal trunks evidently allow adequate ventilation.

The development of experimental animals was assessed by recording morphological changes in the integument and, after 10 days, by investigating the developmental stage of the internal organs (Sehnal, 1968). Development was also estimated in unrestrained larvae ligated behind the prothorax.

Sensitivity of restrained insects to the prothoracotropic hormone and to the molting hormone was checked using larvae of the 6th day of the last larval instar. In the first set of experiments, each larva received an implant of five brains taken from intact insects of the same age. Hosts were allowed to recover for five hours and were then restrained with adhesive tape. One group of larvae received combined brain, corpora cardiaca and corpora allata implants. Controls were implanted with thoracic ganglia or with pieces of gut.

In the second set of experiments, effects of molting hormone were estimated by injecting larvae either with alpha-ecdysone (Huber and Hoppe, 1965) or with ecdysterone (Hoffmeister, 1966). The first compound was synthesized by Syntex Co., California, the second was extracted from the roots of *Polypodium vulgare* (Jizba, Herout and Šorm, 1967). Each larva was injected with 8 μg of one of the substances dissolved in 4 μl of 10% alcohol, and then restrained with adhesive tape. Controls received equal amounts of the solvent alone. Sensitivity of nonrestrained intact of ligated larvae was established in a similar way.

Results

The effects of restraint and of ligature in relation to developmental stage are summarized in Table I. Except for larvae from well-formed cocoons (about one day prior to the larval-pupal ecdysis) all insects remained at the developmental stage reached at the time of restraint. No changes in either integument or internal organs were visible within 10 days of restraint, except for the emptying of the gut and occasional accumulation of uric acid granules in the Malpighian tubules. Starving intact larvae either pupated within 10 days, or died subsequently.

Ligature and restraint had similar effects on development. Only larvae from well-formed cocoons were able to pupate after isolating the brain and the prothoracic glands by ligation.

Implantation of extra brains into restrained larvae was without detectable effect in most cases (Table II). Few specimens initiated development and underwent

TABLE I

Effect of body restraint and of ligature on the development of Galleria *larvae*

Developmental stage	Days of the instar	Restraint		Ligature		Comments
		Total number	Number molted	Total number	Number molted	
	Penultimate larval instar					
Feeding larva	2	10	0	10	0	No developmental progress
	Last larval instar					
Feeding larva	3	15	0	30	0	No developmental progress
Preparation for spinning	5	15	0	30	0	No developmental progress
Onset of spinning	6	15	0	30	0	No developmental progress
Larva in well-formed cocoon	7	25	17	30	15	In affected insects internal organs as in pupae. Limbs, wings, and other organs wihch developed from imaginal discs are reduced in size and structure. Pupal cuticle not always sclerotized.

Controls left inteact but without food. Experiments evaluated 10 days after the respective treatment when majority of controls either died or molted.

a molt. Of those animals which did develop, insects with implanted brains molted into pupae whereas larvae with implanted complexes molted to forms intermediate between larva and pupa (Piepho, 1940). The molting process in the integument was not always completed and the new cuticle often remained untanned.

Unlike the implantations of brains, injections of alpha-ecdysone or ecdysterone were effective in inducing development in restrained animals (Table III). Diges-

TABLE II

Effect of implanted brains on restrained larvae

Developmental stage	Implant	Number of insects	Number molted	Comments
Larva at onset of spinning	5 brains	14	3	Molted into pupae (4 died)
Larva at onset of spinning	5 complexes brain-c. cardiaca-c. allata	14	4	Molted into intermediates between larva and pupa (2 died)

Experiments were evaluated 10 days after treatment. Within this period only one of 25 controls molted. Controls were implanted either with thoracic ganglia or with pieces of gut.

tion of the old cuticle by the molting fluid proceeded to various degrees in different individuals. New cuticle bore pupal characteristics; it was usually well-formed and occasionally tanned, but cell proliferation, which normally accompanies the molting process, was suppressed. Limbs and wings were consequently smaller and morphologically simpler than in normal pupae. Characteristic pupal structures, such as the mid-dorsal rib, were lacking or reduced. Furthermore, gaps developed in epidermis in prolegs and genitalia regions, where larval cells degenerated but new epidermis failed to proliferate from the surroundings.

Ecdysone had an identical effect on larvae ligated behind the prothorax, but in all cases injection of solvent alone did not cause development to proceed in restrained or ligatured larvae.

TABLE III

Effects of ecdysones on intact, ligated, and restrained larvae of Galleria

Developmental stage	Injected:	Total number of insects	Number molted	Number dead
		Intact larvae		
Larva at onset of spinning	8 μg of ecdysterone	10	8	2
		Ligated larvae		
Larva at onset of spinning	8 μg of ecdysterone	10	7	3
Larva at onset of spinning	8 μg of alfa-ecdysone	25	17	5
		Restrained larvae		
Larva at onset of spinning	8 μg of ecdysterone	15	11	4
Larva at onset of spinning	8 μg of alfa-ecdysone	15	10	1

Insects usually molted within 4 days after the injections; remaining larvae were discarded after 10 days. Injection of the solvent alone into the control larvae had no effect.

DISCUSSION

Insect development is regulated by the nervous and endocrine systems. The common scheme for the control of the molting process, which repeats several times in the course of insect ontogeny, involves three steps (reviews by Wigglesworth, 1964; Novak, 1966). (1) Various stimuli, such as light, moisture, temperature, and food, act, probably through nervous pathways, on the neurosecretory system of the brain. Under suitable conditions, which apparently include such internal factors as increase in body size and accomplishment of certain morphogenetic

processes, prothoracotropic hormone (synonyms: activation or brain hormone) is released and activates the prothoracic glands. (2) Prothoracic glands in turn secrete the molting hormone, alpha-ecdysone or ecdysterone. (3) Molting hormone evokes the molting process.

Immobilization of *Galleria* by mechanical restraint as described above suspends development in both penultimate and last larval instars. Animals so treated may survive for long periods without detectable change other than slow depletion of metabolic reserves (Edwards, 1967). The developmental block cannot be due to starvation, because it occurs in larvae that have completed feeding in the last instar, and which have entered the "wandering" stage that leads to cocoon spinning. When feeding larvae are taken from their food they either molt, or die long before their restrained contemporaries die (Sehnal, 1966).

A critical point is passed after the cocoon formation has been completed beyond which restraint no longer suspends development. Larvae at this developmental stage also pupate after ligating off the brain and prothoracic glands. In the earlier period of the instar, when restraint is effective, presence of the brain and prothoracic glands is indispensible for development. This coincidence suggests that bodily constraint hampers the molting process by affecting these glands and thus interrupting hormone production.

Prothoracotropic hormone could theoretically be supplied to restrained larvae by implanting several extra brains. The results obtained with implanted brains were variable: since the molting process was initiated in some animals it is clear that the prothoracic glands remain sensitive to activation and capable of secreting ecdysone. It is well known that implantation of potentially active brains may have little effect on unrestrained larvae which are deprived of their own brains by head ligature (Kuhn and Piepho, 1936; Sehnal, unpublished observations). The variable behavior of isolated brains awaits explanation. It is beyond the scope of the present study, but it seems probable that deficiencies of titer and duration of secretion are involved.

When the action of both brain and prothoracic glands was circumvented by injecting larvae with alpha-ecdysone or ecdysterone, restrained larvae responded by molting. The resulting pupae were not perfect but the molt had all typical features: detachment of the old cuticle, its partial digestion, secretion of a new cuticle and its occasional tanning.

Since larvae which are deprived of brain and prothoracic glands by ligature also respond to ecdysones, the injected hormone evidently acts directly on the target tissues. Sensitivity of the targets does not appear to be affected by restraint; for non-restrained larvae, both intact and ligated, respond to ecdysones in the same way.

Our observations indicate that bodily constraint inhibits secretion by the brain. This supports the earlier assumption that appropriate sensory input from the periphery must precede secretion of the prothoracotropic hormone (Edwards, 1967). Mechanical restraint of the larva apparently modifies the input and thus prevents the whole molting process. The effect can be by-passed by providing the prothoracotropic hormone or molting hormone by injection. Our results show that restraint inhibits development at any time in the course of the last larval instar.

Hence, a secretion from the brain seems to be required for a prolonged period of time and not only for an instantaneous activation of prothoracic glands.

Several other examples in which proprioception has been implicated in the regulation of neuroendocrine events may be briefly noted. Wigglesworth (1934) concluded that distension of the body wall provided the signal for the initiation of the molting cycle in *Rhodnius*. Clarke and Langley (1963) postulated a role for proprioceptive monitoring of crop distension in growth and molting of *Locusta*, and a similar control system has been suggested in the regulation of digestive enzyme activity in adult tsetse flies (Langley, 1966). Finlayson (1967) has recently demonstrated that mechanoreceptor input plays a role in the initiation of puparium formation in larvae of *Glossina*. The onset and termination of successive phases of insect molting and maturation is regulated by surface contact and body form in a number of studies cited by Cottrell (1964) and in certain of these, proprioceptive monitoring of body form is implicit.

Further significant progress toward proof of proprioceptive function as a factor in regulation of certain neurosecretions must await work on the central processing of input from the various proprioceptors now known in insects (Finlayson, 1968), and on the neuronal connections with neurosecretory cells for which there now is neither neuroanatomical nor physiological data.

Summary

Restraint of *Galleria* larvae by mechanical means suspends the capacity for development. Restraint inhibits, apparently by a nervous route, secretion of prothoracotropic hormone from the brain; prothoracic glands do not produce the molting hormone and larvae thus never molt. Targets of the molting hormone are not affected and respond normally to an artificial supply of the hormone. These results support the hypothesis that appropriate proprioceptive input is a necessary condition for the normal activation of the neuro-endocrine system.

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